

Eucaryotic Cells Protect *Borrelia burgdorferi* from the Action of Penicillin and Ceftriaxone but Not from the Action of Doxycycline and Erythromycin

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Despite appropriate antibiotic treatment, Lyme disease patients may have relapses or may develop chronic manifestations. The intracellular location of *Borrelia burgdorferi* suggests that antibiotics that penetrate cells will have greater efficiency. Doxycycline or erythromycin was more effective than penicillin or ceftriaxone in killing *B. burgdorferi* when the organism was grown in the presence of eucaryotic cells.

At present the treatment of Lyme borreliosis, a disease caused by the spirochete *Borrelia burgdorferi*, poses several problems. The antibiotics usually recommended for the treatment of Lyme borreliosis are amoxicillin, doxycycline, erythromycin, penicillin G, and ceftriaxone (7). However, the failure of antibiotics in treating Lyme disease in humans has been reported several times (2, 6, 9). Relapses of neuroborreliosis along with the isolation of ceftriaxone-susceptible spirochetes from cerebrospinal fluid have been observed following ceftriaxone treatment (6), suggesting that the antibiotic could not gain access to *B. burgdorferi*, leading to the hypothesis that the organism has an intracellular location. Previous studies have demonstrated the attachment (10), penetration (1), and intracellular localization (4) of *B. burgdorferi* isolates. Georgilis et al. (3) demonstrated the protective effects of human fibroblasts and Vero and HEp-2 cells on *B. burgdorferi* against the action of ceftriaxone. This strongly suggests that *B. burgdorferi* might dissimulate within certain host cells and therefore escape the host immune response and the actions of antibiotics.

Consequently, one may suggest that only use of an antibiotic with a good intracellular distribution might allow for definitive sterilization. We demonstrate here that the protective effects of eucaryotic cells against the actions of penicillin and ceftriaxone were absent when doxycycline and erythromycin were used.

B. burgdorferi B 31 (ATCC 35210), at the 32nd passage, was obtained from G. Baranton (Institut Pasteur, Paris, France), and was cultured at 32°C in modified Barbour-Stoenner-Kelly (BSK) medium. Human endothelial cells (ECV 304.9 cells; Cerdic, Valbonne, France) and Vero cells (ATCC CRL 6318) were cultivated in RPMI 1640 medium–10% fetal bovine serum (FBS; Eurobio, les Ulis, France)–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Sigma, St. Louis, Mo.)–1% L-glutamine in a 5% CO₂ atmosphere. The antibiotics tested were penicillin G (Diamant, Puteaux, France), doxycycline (Pfizer, Orsay, France), erythromycin (Abbott, Rungis, France), and ceftriaxone (Roche, Neuilly-sur-Seine, France). After 48 h of cell culture, the supernatant was removed and was replaced with 100 µl of BSK medium containing 10⁷ *B. burgdorferi* organisms at the logarithmic phase of

growth and 100 µl of cell culture medium (RPMI 1640, 10% FBS, 1% L-glutamine, 10 mM HEPES). The *B. burgdorferi* isolates cocultivated with cells were incubated at 32°C in a 5% CO₂ atmosphere, whereas the *B. burgdorferi* isolates cultivated in axenic medium were incubated at 32°C without CO₂. After 48 h of incubation of the *B. burgdorferi* isolates, a mixture of 100 µl of BSK medium and 100 µl of cell culture medium containing antibiotics was added to obtain a final concentration of 16 µg/ml for penicillin G, 8 µg/ml for doxycycline, 4 µg/ml for erythromycin, and 32 µg/ml for ceftriaxone. As a control, antibiotic-free medium was added. All plates were then reincubated for 72 h under the conditions described above.

Inoculum titration was carried out at the time of antibiotic addition (time zero) and at 24, 48, and 72 h postincubation with antibiotics by using a limiting dilution procedure in 96-well microtiter plates (Nunc). To assess cell-associated bacteria only, the supernatant was first removed. The infected cells were then washed twice with phosphate-buffered saline (PBS), resuspended in 200 µl of BSK medium–RPMI 1640 (vol/vol) and harvested, and the bacteria were mechanically released from the cell through a 27-gauge needle. Samples were then dispensed into the wells of 96-well microtiter plates, serial dilutions from 10⁻¹ to 10⁻⁹ in BSK medium were made, and the plates were incubated for 6 days. After 6 days of incubation, bacterial growth was evaluated by dark-field microscopy. Each experiment was done two times to verify the reproducibility. Results were expressed as the variation in the number of residual viable bacteria ($\log N - \log I$)/ $\log I$, where *N* is the number of bacteria counted in the experiment and *I* is the number of bacteria in the inoculum.

No change in the residual viable bacterial count was observed between day 0 and day 3 when *B. burgdorferi* was cocultivated with eucaryotic cells in the presence of penicillin G (Fig. 1A), reflecting the protective effect of cells in this situation. On the other hand, in axenic medium, the bacterial count decreased rapidly. Similar results were noted with ceftriaxone-treated *B. burgdorferi* isolates (Fig. 1B). Erythromycin was found to act very efficiently against *B. burgdorferi*, especially when the organism was grown in the presence of eucaryotic cells (Fig. 1C). No viable bacteria were found after 72 h of incubation with this compound. The same observation was noted with doxycycline-treated spirochetes, except that doxycycline seemed to act more rapidly because no bacteria re-

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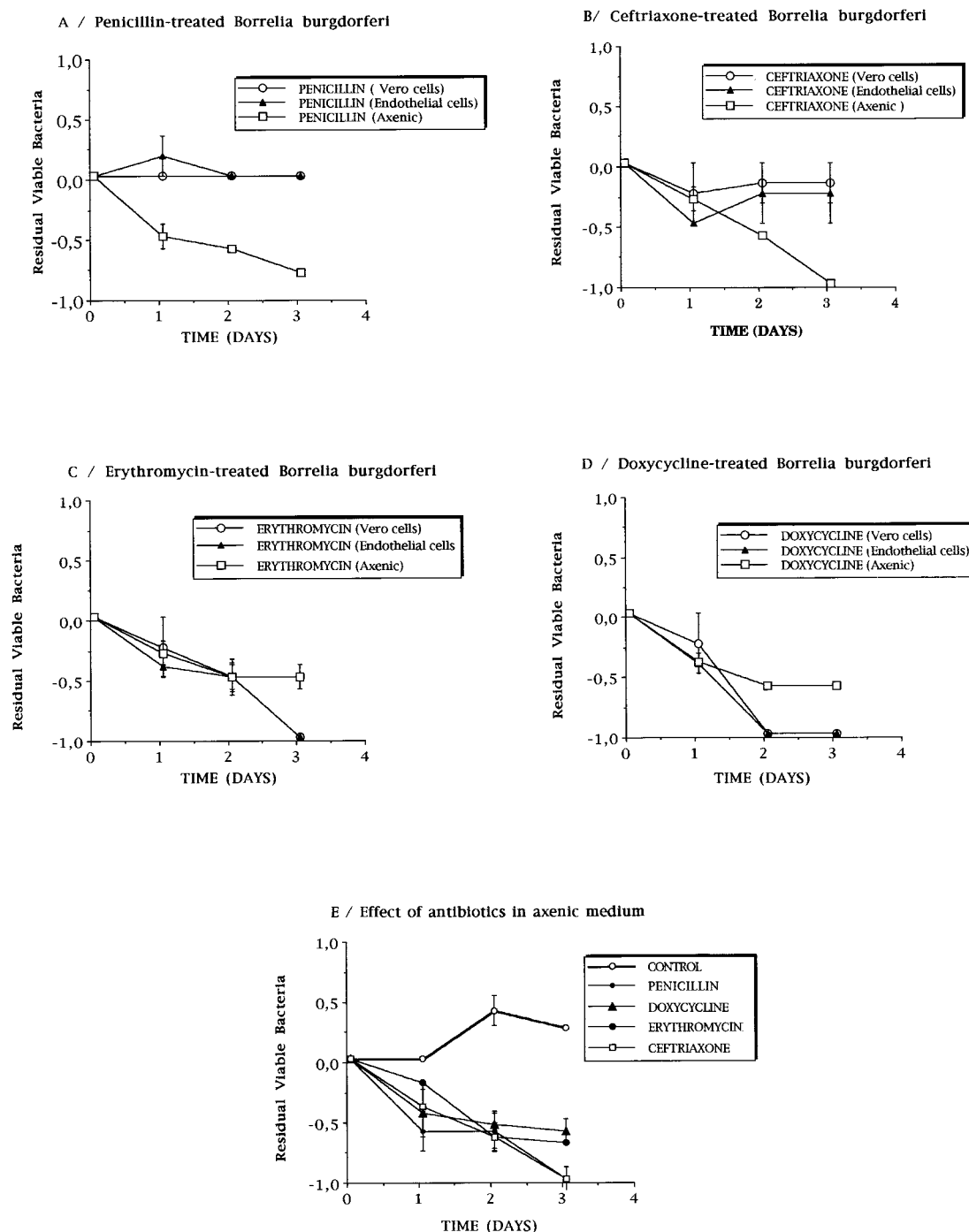


FIG. 1. Residual viable bacteria expressed as a variation in the number of bacteria by the formula $(\log N - \log I)/\log I$. Error bars indicate the standard deviation of the mean variation for two experiments.

mained viable at day 2 of incubation (Fig. 1D). All antibiotics were found to act efficiently in axenic medium, with penicillin and ceftriaxone proving to be the most effective (Fig. 1E). No differences in the results obtained with the two cell lines were observed in these experiments.

The treatment of Lyme borreliosis poses several problems, the most significant of which is the inexplicable occurrence of relapses or chronic symptoms in patients apparently treated correctly. Amoxicillin, doxycycline, ceftriaxone, erythromycin,

or penicillin G is usually prescribed for the treatment of Lyme disease (7). Our results with organisms grown in axenic medium are similar to the previously reported results (7) showing the greater efficiencies of ceftriaxone and penicillin compared with those of doxycycline and erythromycin.

The intracellular residence of a bacterium offers protection from both host defense mechanisms and antibiotics. *B. burgdorferi* isolates adhere to, invade, and survive in human endothelial cells (1, 4, 10). In vivo *B. burgdorferi* has been observed

in cardiac myocytes of experimentally infected mice (5). In our study, while penicillin G and ceftriaxone appeared to be very efficient in axenic medium, they lost their efficiencies when *B. burgdorferi* was cultivated in the presence of cells, confirming the protective effects of cells from the actions of ceftriaxone and penicillin (3).

When *B. burgdorferi* was cultivated in the presence of cells, the cell count was reduced dramatically by erythromycin and doxycycline, indicating their superiority in this situation. Our study included only one strain of *B. burgdorferi*, and one may suggest that the relapse observed with tetracycline (9) may be due to different strain susceptibilities or may be related to antibiotic diffusion, especially in the central nervous system. The killing of obligate or facultative intracellular bacteria would be necessary to avoid relapses in some infectious diseases (8), and the enhanced activities of doxycycline and erythromycin when *B. burgdorferi* is cocultivated with eucaryotic cells demonstrated in our study suggest that clinical trials with tetracycline versus ceftriaxone along with evaluations of patients with long-term relapses are needed.

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